Targeted disruption of growth hormone receptor interferes with the beneficial actions of calorie restriction

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Reduced intake of nutrients [calorie restriction (CR)] extends longevity in organisms ranging from yeast to mammals. Mutations affecting somatotropic, insulin, or homologous signaling pathways can increase life span in worms, flies, and mice, and there is considerable evidence that reduced secretion of insulin-like growth factor I and insulin are among the mechanisms that mediate the effects of CR on aging and longevity in mammals. In the present study, mice with targeted disruption of the growth hormone (GH) receptor [GH receptor/GH-binding protein knockout (GHRKO) mice] and their normal siblings were fed ad libitum (AL) or subjected to 30% CR starting at 2 months of age. In normal females and males, CR produced the expected increases in overall, average, median, and maximal life span. Longevity of normal mice subjected to CR resembles that of GHRKO animals fed AL. In sharp contrast to its effects in normal mice, CR failed to increase overall, median, or average life span in GHRKO mice and increased maximal life span only in females. In a separate group of animals, CR for 1 year improved insulin sensitivity in normal mice but failed to further enhance the remarkable insulin sensitivity in GHRKO mutants. These data imply that somatotropic signaling is critically important not only in the control of aging and longevity under conditions of unlimited food supply but also in mediating the effects of CR on life span. The present findings also support the notion that enhanced sensitivity to insulin plays a prominent role in the actions of CR and GH resistance on longevity.

insulin-like growth factor I | insulin | longevity | aging | dietary restriction

Mutations affecting somatotropic and/or insulin signaling can produce a marked increase of longevity in mice. Genes related to homologous signaling pathways in the yeast *Saccharomyces cerevisiae*, the worm *Caenorhabditis elegans*, and the fly *Drosophila melanogaster* play a key role in the control of aging in these species (1–3). A moderate reduction in the intake of nutrients [also known as calorie restriction (CR)] is extremely effective in delaying aging and increasing longevity in organisms ranging from yeast to mammals (3–5). We have previously reported that CR produces an additional increase in the life span of a long-lived hypopituitary mutant mouse, the Ames dwarf, and alters the slope of its survival curve similarly to the effects of CR in normal mice (6). This result was counterintuitive because both Ames dwarfs and normal animals subjected to CR have reduced insulin-like growth factor I (IGF-I) and insulin levels and share other phenotypic characteristics. Although *C. elegans* with a mutation in the insulin/IGF-I homologous signaling pathway Daf 16/FOXO lived longer when subjected to CR (7, 8), CR failed to further increase longevity in *D. melanogaster* with a *chico* mutation that interferes with insulin/IGF-I signaling and prolongs life (9). Interpretation of the findings obtained in Ames dwarf mice is complicated by the fact that in addition to growth hormone (GH) deficiency, these animals are deficient also in prolactin and thyrotropin (10, 11), and the interaction of CR with reduced lactogenic and/or thyroid hormone signaling could conceivably contribute to the results. It was therefore of interest to examine the effects of CR in a different long-lived mouse mutant in which the primary defect in endocrine function is limited to the somatotropic axis.

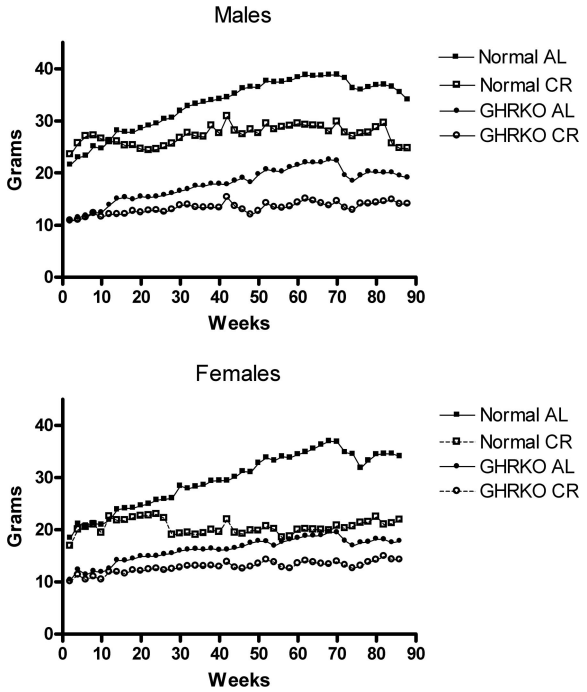

Fig. 1. Time course of changes in body weight for male and female normal and GHRKO mice that were fed AL or subjected to 30% CR. Animals were weighed weekly starting at 2 months of age.

GH receptor/GH-binding protein knockout (GHRKO) mice were developed by Zhou *et al.* (12) by targeted disruption of the *Ghr/Ghrbp* gene. These mutant mice do not express the GH receptor, are GH resistant, and have profoundly suppressed circulating levels of IGF-I and insulin, markedly increased life span, and multiple indices of delayed aging, including increased mortality rate doubling time (12–16).

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Abbreviations: AL, ad libitum; CR, calorie restriction; GH, growth hormone; GHRKO, GH receptor/GH-binding protein knockout; IGF-I, insulin-like growth factor I; ITT, insulin tolerance test.

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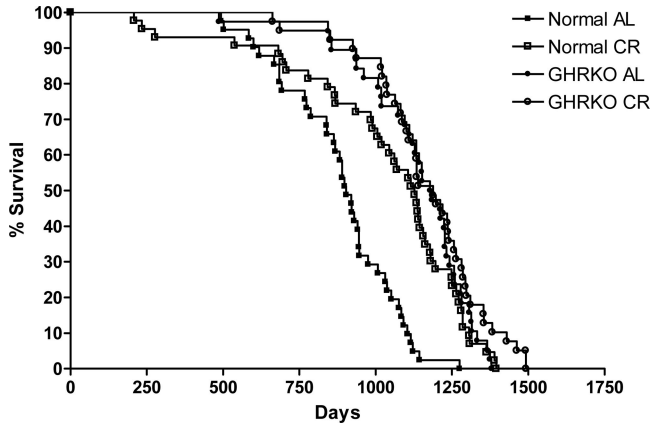

Fig. 2. Kaplan–Meier survival plot of normal and GHRKO mice that were fed AL or subjected to 30% CR starting at 2 months of age and maintained for the remainder of life span. Data from males and females are combined.

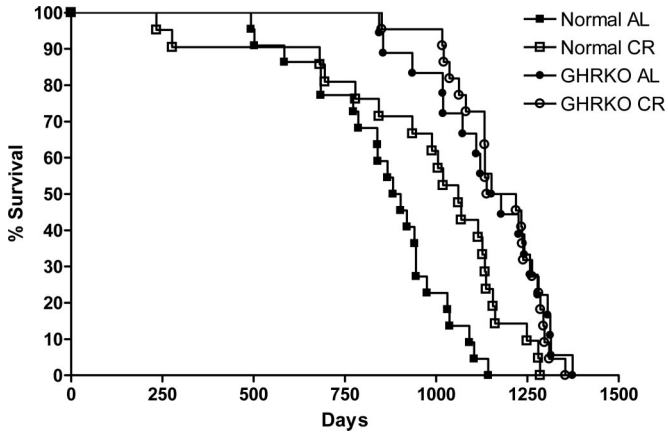

Fig. 3. Kaplan–Meier survival plot of male normal and GHRKO mice that were fed AL or subjected to 30% CR starting at 2 months of age. CR was maintained throughout the life span.

Results

CR was started at 2 months of age and continued throughout the remainder of the study, producing the expected reduction in body weight in both normal and GHRKO mice (Fig. 1). In males, the relative decrease in body weight in response to CR was comparable in normal and GHRKO mice, whereas in females, the response to CR was more pronounced in normal animals than in GHRKO animals.

As expected, under conditions of ad libitum (AL) feeding, GHRKO mice lived much longer than the normal (control) animals. Analysis of longevity data collapsed across sexes revealed that, in normal mice, CR produced the expected significant increase in overall survival (log-rank test) and average, median, and maximal longevity (Fig. 2 and Table 1). In contrast, CR did not affect the overall survival or average or median longevity of GHRKO mice and produced a smaller (7.8% in GHRKO mice vs. 17.2% in normal mice) although statistically significant increase in the maximal longevity of GHRKO animals. It was particularly striking that median longevity was increased by 25% in normal-CR mice compared with normal-AL mice, and it was nearly identical (1,188 vs. 1,181 days) in GHRKO-AL and GHRKO-CR mice.

When male and female longevity data were analyzed separately, it became evident that the effects of CR on maximal longevity were sexually dimorphic in GHRKO mice but not in normal mice. In normal males (Fig. 3), CR increased overall and maximal survival, and median longevity was increased by 18.8%. In GHRKO males, CR did not affect overall, average, or maximal longevity, whereas median longevity was increased by 1.2%. In normal females (Fig. 4), CR increased each of the examined parameters, including a 28% increase in median longevity. In GHRKO females, CR did not affect overall, average, or median longevity (median longevity was 1% shorter in GHRKO-CR mice than in GHRKO-AL mice) but produced a significant increase in maximal longevity. The increase in maximal longevity was proportionally smaller than that mea-

Table 1. Longevity characteristics in normal and GHRKO mice that were fed AL or subjected to 30% CR

	Phenotype			
Measures of longevity	Normal-AL	Normal-CR	GHRKO-AL	GHRKO-CR
Males and females				
Log-rank test of survival curves	*	†	†	†
Average	887 ± 29*	1,028 ± 45†	1,139 ± 30‡	1,167 ± 31‡
Median	903	1,127	1,181	1,188
Maximal	1,163 ± 38*	1,363 ± 20†	1,362 ± 11†	1,468 ± 15‡
Males only				
Log-rank test of survival curves	*	†	‡	‡
Average	862 ± 39*	963 ± 64*	1,145 ± 38†	1,171 ± 27†
Median	893	1,061	1,165	1,179
Maximal	1,113 ± 16*	1,271 ± 11†	1,334 ± 20†	1,320 ± 17†
Females only				
Log-rank test of survival curves	*	†	†	†
Average	916 ± 43*	1,090 ± 63†	1,133 ± 46†	1,154 ± 63†
Median	921	1,179	1,198	1,188
Maximal	1,158 ± 59*	1,382 ± 10†	1,359 ± 14†	1,481 ± 10‡

All data are expressed as mean days ± SEM (median when noted). Log-rank test of survival curves labeled with different symbols are significantly different (see Figs. 2–4). In each row, values with unlike superscript symbols are significantly different ($P < 0.05$).

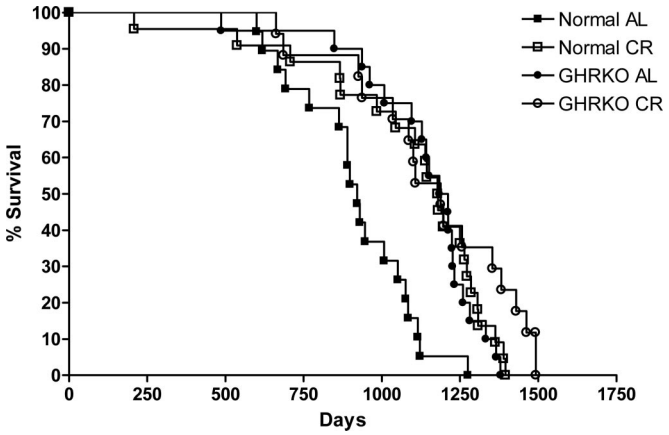

Fig. 4. Kaplan–Meier survival plot of female normal and GHRKO mice that were fed AL or subjected to 30% CR starting at 2 months of age. CR was maintained throughout the life span.

sured in normal females (9% in GHRKO females vs. 19% in normal females).

In a separate group of normal and GHRKO mice, 30% CR for 1 year produced the expected decrease in body weight that was statistically significant in each sex/genotype group (data not shown). Blood glucose levels were measured in samples collected before and 15, 30, and 60 min after insulin injection. In normal male and female mice, CR significantly increased sensitivity to insulin challenge. Insulin sensitivity in GHRKO animals was greater than in normal mice and was not further increased by CR in either sex (Fig. 5).

Discussion

The main finding of the present study is the striking difference in the response of GHRKO and normal mice to an identical regimen of CR. In normal mice, 30% CR produced the expected robust increase in longevity. GHRKO mice that were fed AL lived much longer than normal mice and thus resembled normal CR animals. Subjecting GHRKO mice to CR failed to produce any further extension of overall, median, or average longevity. Maximal longevity of GHRKO mice, estimated by the mean age of death of the oldest 10% in each group, was extended by CR very modestly and only in females. Although the magnitude of the effect of CR on life span is strain-dependent (17), failure to observe life extension in CR mice is extremely rare if not unprecedented (18). In particular, the present findings in GHRKO mice contrast sharply with significant extensions of longevity in normal (control) animals from the same line (Table 1 and Fig. 2). These findings also differ from the results obtained in long-lived Ames dwarfs, which share many phenotypic characteristics with GHRKO mice and were exposed to an identical CR protocol in an earlier study in our laboratory (6). Both GHRKO and Ames dwarf mice are characterized by extremely low levels of IGF-I in peripheral circulation, reduced postnatal growth, delayed puberty, diminished body size (19), reduced plasma insulin and glucose, and enhanced sensitivity to injected insulin (20, 21). Because normal siblings of GHRKO mice exhibited a robust longevity response to CR in the present study, it appears exceedingly unlikely that differences in the responses of Ames dwarf and GHRKO mice to CR are due to distinct genetic backgrounds or differences in some hard-to-define environmental factors between studies conducted several years apart.

Phenotypic differences between GHRKO and Ames dwarf mice include opposite alterations in prolactin levels; varying degrees of suppression of thyroid hormone levels and body core temperature; major differences in reproductive status, particularly in females; and differences in body composition, antioxidant enzymes, and end-of-life pathology (19, 22). From the present data, we cannot conclude which of these differences may have contributed to their divergent responses to 30% CR.

Absence of additional extension of life by CR in GHRKO animals in the present study could have been due to the particular regimen of CR chosen or to the "ceiling effect" (i.e., maximal life extension) in GHRKO animals that were fed AL. Clancy *et al.* (9) elegantly demonstrated that, in *Drosophila*, different severities of CR are optimally effective for life extension in wild-type and in long-lived mutant flies. Although we do not know whether a different (milder) regimen of CR may have allowed GHRKO mice to respond by life extension, the 30% CR restriction used in the present study is not considered severe.

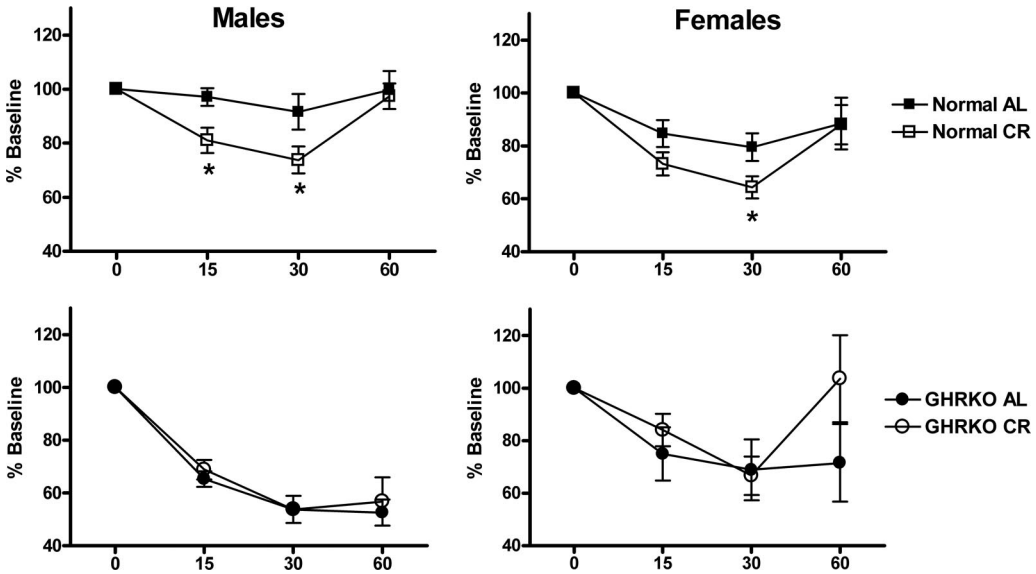

Fig. 5. Results of ITT in normal and GHRKO mice that were fed AL or subjected to 30% CR between 2 and 12 months of age. All groups were randomly fed (100% AL) overnight. Mice were injected i.p. with insulin (0.75 units/kg of body weight). Glucose was measured in samples collected from the tail vein at specified time points. *, $P < 0.05$ compared with AL controls within phenotype.

Moreover, an identical regimen of CR was previously shown to extend life span in another long-lived mutant that resembled GHRKO mice in body size, insulin sensitivity, and other characteristics (ref. 6; detailed above).

It is exceedingly unlikely that GHRKO mutation produces the maximal possible life extension and thus that CR in animals of this genotype causes near starvation rather than a beneficial reduction in nutrient intake. Autopsy findings and studies of body composition indicate that GHRKO-CR mice are not depleted of adipose tissue. Extrinsic factors including starvation unmistakably alter the shape of a survival curve (17, 23), and there was no indication of such alterations in GHRKO-AL and GHRKO-CR mice. In fact, 90% of the GHRKO-CR mice remained alive at 925 days of age (Fig. 2).

An important implication of the present findings is that targeted disruption of the GH receptor and the resulting GH resistance mimic some of the effects of CR in mice, although numerous differences also exist (24, 25). Moreover, these findings imply that the GH receptor or GH receptor-dependent GH signaling is extremely important and may possibly be required for CR to extend mammalian longevity. Thus, CR-induced suppression of the somatotropic axis emerges as one key (or perhaps the key) candidate for mechanisms linking CR with increased longevity.

It is also interesting to view the present results in a broader context of interactions between "longevity genes" and CR. Studies in *C. elegans* suggest that some of the mutations that extend life act through mechanisms similar and perhaps identical to those that are responsible for the effects of CR (7) but that genes related to the insulin-like pathway do not (7, 8). Results obtained from *Drosophila* suggest considerable similarity between the actions of CR and genes related to the insulin-like signaling pathway, although outcomes of specific studies may depend on the severity of CR imposed (9). In the mouse, previous studies in Ames dwarf mice suggested that this mutation ($Prop1^{df}$) and CR affect longevity by distinct but likely overlapping mechanisms (6). In support of this conclusion, the effects of $Prop1^{df}$ and CR on life span are additive, whereas their effects on the slope of the survival plot are different (6). In contrast, close similarity of survival of normal-CR, GHRKO-AL, and GHRKO-CR mice in the present study would suggest that targeted disruption of the *Ghr/Ghrbp* gene and CR affect longevity by the same mechanism. This conclusion was not expected from our earlier studies that identified major differences in the effects of CR and GHRKO mutation on a wide profile of hepatic gene expression (24) and on the expression of selected insulin- and IGF-I-related genes in the liver (25), skeletal muscle (26), and heart (27). However, in support of the present findings, analysis of liver gene expression revealed a significant interaction between CR diet and GHRKO mutation, whereas significantly fewer genes were altered by the CR regimen in GHRKO mice compared with normal control littermates (24).

On the basis of the present and previous results, we suggest that the failure of CR to increase overall, median, or average longevity in GHRKO mice is related to its failure to improve insulin sensitivity in these mutant animals. There are numerous indications that insulin and insulin release and actions play a major role in the control of mammalian aging. We and others have previously suggested that increased sensitivity to insulin (measured directly or implied by concomitant reductions in plasma insulin and glucose levels) accounts for, or importantly contributes to, extended longevity of Ames dwarf (21), Snell dwarf (28), and GHRKO (20) animals as well as mice exposed to CR (29). In an attempt to identify reasons for the failure of CR to extend longevity of GHRKO mice, we examined the responses to injected insulin [the so-called insulin tolerance test (ITT)] in a separate group of GHRKO and normal mice subjected to an identical CR regimen for 12 months (Fig. 5). CR produced the expected improvement in insulin sensitivity of normal mice. Insulin sensitivity of GHRKO mutants was greatly increased in comparison with normal mice, as expected from previous studies (21), and was not further enhanced by CR. We have previously reported that in GHRKO mice, but not in normal mice, CR enhances the expression of hepatic genes related to gluconeogenesis and fails to further reduce hepatic levels of phosphorylated Akt, an important mediator of insulin action (25). More recently, we reported that liver expression of insulin receptor and insulin receptor substrate 2 were elevated in normal-CR, GHRKO-AL, and GHRKO-CR mice when compared with normal-AL control animals (26).

A recent report of increased life span in transgenic Klotho mice that are insulin-resistant (30) raises an interesting possibility: that aging can be delayed by reduced strength of the insulin signal, regardless of its underlying causes (31). Thus, effects of mild insulin resistance in Klotho transgenics and adipose-specific insulin resistance in FIRKO (fat-specific insulin receptor knockout) mice (32) may overlap the effects of reduced insulin and IGF-I release in GHRKO, dwarf, and CR animals.

We conclude that, in the absence of a functional GH receptor, CR does not affect most of the examined measures of longevity in mice. The failure of CR to increase average life span of GHRKO mice or maximal life span of GHRKO males was associated with its failure to further increase insulin sensitivity in these animals. We suspect that the divergent effects of CR on gene expression and levels of signaling molecules in GH and insulin target organs may underlie differences between normal and GHRKO mice in deriving a longevity benefit from CR.

A broader implication of the present findings is that insulin signaling emerges as an important determinant of mammalian aging and longevity. This implication raises the issue of the potential (and, we believe, likely) impact of insulin-resistant states, including the current "epidemic" of metabolic syndrome on life expectancy in this society and others. We suspect that research efforts to develop "CR mimetics" for pharmacological intervention in the aging process may be more productive if they focus on targets in the GH/IGF-I/insulin signaling axis.

Materials and Methods

Animals. GHRKO and normal mice were produced in our breeding colony derived from GHRKO animals kindly provided by J. J. Kopchick (Ohio University, Athens). Phenotypically normal siblings of GHRKO mice served as controls for this study. Animals were housed under temperature- and light-controlled conditions (20–23°C and 12-h light/12-h dark cycle) and were fed Lab Diet Formula 5001 (Ralston Purina). Sentinel animals were sent for bacterial and viral testing every 3 months, and the results were uniformly negative. All animal protocols for this study were approved by the Animal Care and Use Committee of Southern Illinois University.

Longevity Study. All animals (n = 37–43 per genotype per diet group) were studied beginning at 8 weeks of age. In both longevity and long-term (1 year) studies, animals were gradually placed on 30% CR by receiving 90% of the amount of food consumed by AL controls in the initial week, 80% the following week, and 70% throughout the rest of the studies. Animals in the longevity study were checked daily for health and survival and were handled for cage changes and weekly body weight measurements only. Animals that appeared to be near death (listless, unable to walk, and cold to the touch) or had large bleeding tumors or neoplastic growth approaching 10% of body weight were euthanized, and the date of euthanasia was considered the date of death. As of this writing, 147 animals had died, 13 had been euthanized, and 1 was still alive.

ITT. In a separate group of mice, CR was imposed as described above. After 12 months of CR, ITTs were conducted. All groups (n = 8–10 per genotype per diet per sex) were randomly fed (100% AL) the night before the test. The following morning, food was removed, and basal glucose was determined by using a glucometer (Lifescan; Johnson & Johnson, New Brunswick, NJ) in blood obtained by removing the tip of the tail. Porcine insulin (Sigma) was injected i.p. at 0.75 units/kg of body weight. Blood was subsequently sampled at 15, 30, and 60 min thereafter for glucose measurements.

Statistical Analysis. Kaplan–Meier survival curves were used for survival analysis, with a log-rank test to evaluate significance of differences between groups. Median life span represents the age at which 50% of the population within groups remained alive. Maximal life span was calculated as the mean age of the oldest 10% of the population within that group, and significance was tested by using two-way ANOVA and independent t tests. Results of ITTs were plotted as mean percentage change from baseline within experimental groups. Repeated measures ANOVA was used to determine the interaction of the main effect variables, phenotype and diet. Independent t tests were used to detect significant differences at specified time points. All graphs were developed by using PRISM 4 (GraphPad, San Diego). All statistical analyses were conducted by using SPSS 12 (SPSS, Chicago). α was set at 0.05 for determination of significance. With the exception of log-rank longevity data, all values are reported as mean ± SEM throughout the figures and text.

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